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Analysis of different pigmentation patterns in 'Mantianhong' (*Pyrus pyrifolia* Nakai) and 'Cascade' (*Pyrus communis* L.) under bagging treatment and postharvest UV-B/visible irradiation conditions

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ABSTRACT

The pigmentation patterns of 'Mantianhong' (*Pyrus pyrifolia* Nakai) and 'Cascade' (*Pyrus communis* L.) that develop under bagging and postharvest UV-B/visible irradiation conditions were evaluated in this study. The results showed that, unlike 'Cascade,' in which almost no anthocyanin was detected, 'Mantianhong' developed good red coloration under bagging treatment and postharvest irradiation. The activity of PAL and DFR increased during anthocyanin accumulation. The expression of *PpCHS3*, *PpCHI3*, *PpUFGT1*, and *PyMYB10* was up-regulated in 'Mantianhong' but not in 'Cascade' during both treatments. This suggests that these four genes may be limiting factors for peel coloration in 'Cascade.' The expression patterns of gene family members were also different during fruit red coloration, suggesting that different gene family members may have distinct functions during pear development. The results obtained in this study are helpful in understanding the molecular mechanism of anthocyanin accumulation in red pears and can serve as the basis for the development of marker-assisted selection or for anthocyanin metabolic engineering of pears.

1. Introduction

Pears are a main pome fruit in temperate regions. Chinese sand pears (*Pyrus pyrifolia* Nakai) and European pears (*Pyrus communis* L.) are the world's two main commercial pear species (Bell et al., 1996). Chinese sand pears, which are predominantly cultivated in China, Korea, and Japan, commonly appear green, yellow, or russet-brown (Teng and Tanabe, 2004). Recently, some red sand pear cultivars have been discovered in China (Huang et al., 2009). Europe, North and South America, Africa, and Australia are the main places where European pears are cultivated, and these pears usually develop a red pigmentation in their skins (Feng et al., 2011). The red coloration of pear skin is mainly due to the concentration and composition of anthocyanin (Steyn et al., 2005). Anthocyanin is a kind of plant pigment and it shows significant antioxidant potential in healthy human nutrition, which results in red and purple colored fruits, vegetables and new cultivars of other important crops for food industry such as potatoes being highly appreciated due to their anthocyanin content (Lachman et al., 2000, 2009). The two predominant anthocyanin pigments in pears are cyanidin-3-galactoside and cyanidin-3-arabinoside (Dussi et al., 1995). Although both Chinese sand pears and European pears have red-skinned cultivars, they differ considerably in the pigmentation patterns. Unlike red Chinese sand pears, in which anthocyanin accumulation peaks in mature fruit (Huang et al., 2009), European pears show a reduction in red coloration as harvest approaches. In European pears, anthocyanin accumulation peaks about midway between anthesis and harvest (Steyn et al., 2005).

The biosynthetic pathway of anthocyanin has been well established in many plant species, such as petunia, snapdragon, and maize (Grotewold, 2006; Holton and Cornish, 1995; Winkel-Shirley, 2001). Genes related to anthocyanin synthesis in pears have also been cloned (Feng et al., 2010; Fischer et al., 2007; Yu et al., 2012; Zhang X.D. et al., 2011). These include five structural genes encoding enzymes directly catalyzing the reactions of anthocyanin biosynthesis: phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT), and three regulatory genes: *MYB10*, *bHLH*, and *WD40*.

Fruit bagging and postharvest ultraviolet-B (UV-B)/visible irradiation are considered attached and detached treatments. They have both been found to improve fruit color. Fruit bagging, has

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also been used to induce anthocyanin biosynthesis and ameliorate fruit coloration in apples and Asian pears (Arakawa, 1988; Huang et al., 2009). Postharvest irradiation has been widely applied to apples, Asian pears, grapes, blackberries and cherries (Basiouny, 1998; Kataoka et al., 2005; Li et al., 2009; Ubi et al., 2006; Zhang D. et al., 2012).

The response of red Chinese sand pears to fruit bagging treatment and postharvest irradiation has been well documented (Huang et al., 2009; Yu et al., 2012; Zhang D. et al., 2012). However, few studies have focused on the influence of attached and detached stimulation on anthocyanin accumulation in European pears. In 'Forelle' pears, a 30-day re-exposure to light before harvest led to the development of good red coloration (Marais et al., 2001). However, knowledge of the effects of short re-exposure before harvest on anthocyanin accumulation in European pears is still lacking. It has been reported that postharvest irradiation cannot enhance anthocyanin biosynthesis in European pears. This is probably because of the acrylic layer between the light source and the fruit, which absorbs the UV light (Marais et al., 2001). The effects of UV-B/visible light exposure on European pears remain unknown.

Members of anthocyanin biosynthetic gene family have been found to be involved in pigmentation pattern in various species. In grapes, three CHS_s (*CHS1*, *CHS2*, and *CHS3*), two CHI_s (*CHI1* and *CHI2*), and two $F3H_s$ (*F3H1* and *F3H2*) have been isolated. The expression of these genes was found to differ significantly in grape berry skins during the coloration process (Jeong et al., 2004). Two PAL_s, *RiPAL1* and *RiPAL2*, were cloned in raspberries and found to share 88% amino acid sequence similarity (Kumar and Ellis, 2001). In apples, F3′H was encoded by a family of two genes (*MdF3′HI*, *MdF3′HII*) (Han et al., 2010). It has also been reported that a total of 10 *CHS* genes have been isolated from *Petunia hybrida* (Koes et al., 1989). In pears, using RACE techniques, two PAL_s (*PpPAL1* and *PpPAL2*), four CHS_s (*PpCHS1*, *PpCHS2*, *PpCHS3*, and *PpCHS4*), three CHI_s (*PpCHI1*, *PpCHI2*, and *PpCHI3*), one *F3H* (*PpF3H*), two DFR_s (*PpDFR1* and *PpDFR2*), one *ANS* (*PpANS*) and two $UFGT_s$ (*PpUFGT1* and *PpUFGT2*) were isolated from red Chinese sand pears (Yu, 2012).

To determine the different pigmentation patterns between red Chinese sand pears and European pears, fruit color, total anthocyanin content, and the expression analysis of a total of 18 related anthocyanin biosynthetic genes were analyzed across two pear cultivars, 'Mantianhong' (*P. pyrifolia*) and 'Cascade' (*P. communis*). Both cultivars were subjected to bagging treatment and postharvest irradiation.

2. Materials and methods

2.1. Fruit materials and experimental treatments

The red Chinese sand pear cultivar 'Mantianhong' was obtained from a commercial orchard in Xingyang City, Henan Province, China and the European pear cultivar 'Cascade' was from the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou City, Henan Province, China. For fruit bagging treatment, 3 mature 'Mantianhong' trees and 3 mature 'Cascade' trees were selected. These trees were similar in size and number of fruit and had uniform exposure to sunlight. Ninety fruitlets from each tree were covered with double layers of yellow-black paper bags [Kobayashi (Qingdao) Co., Ltd., China] 20 days after full bloom. Fruits were re-exposed to sunlight as the bags were removed 10 days before harvest. The fruits that remained bagged served as control samples. Fruits were harvested 0 and 10 days after bag removal (DABR), stored in dry ice, and carried back to the lab as soon as possible. After assessment of fruit color, skins from 5 individual fruits were pooled, instantly frozen in liquid nitrogen and stored at −80 °C for assays.

For the postharvest irradiation experiment, 3 similar trees from each cultivar were chosen. Ninety mature fruits from each tree were given the bagging treatment described above but not re-exposed to light. Two hundred uniform, defect-free fruits per cultivar were divided into two random groups and then were kept under overhead light plant growth chambers (Hangzhou Zeda Instruments Co., Ltd., AGC-D002Z, Hangzhou, China) at 17 °C. One group was used for UV-B/visible irradiation and the other group was stored in the dark environment as a control. UV-B and white light were generated by two UV lamps (PHILIPS PL-S 9W/12 RS, 290–315 nm, Amsterdam, Holland) and four fluorescent tubes (FSL T_8 36W/765, Foshan, Guangdong, China), respectively.

The photon flux density (PFD) at the bottom of the incubator (0.8 m from the light source) was measured using a TES1332A quantum meter (TES Taiwan, China). It reached 252 μmol m^{-2} s^{-1}. Fruits kept in incubators without irradiation served as controls. Samples were collected after 0, 2, 5, 8, and 10 days of irradiation (DI) for assessment of fruit color, extraction of anthocyanin, and analysis of gene expression.

2.2. Assessment of fruit color

The most deeply colored region of the fruit skin was measured using a colorimeter (MiniScan XE Plus, HunterLab, U.S.). CIE L^*, a^*, and b^* values were obtained and transformed into a hue angle degree ($h^o = \arctan[b^*/a^*]$) and chroma ($C = [(a^*)^2 + (b^*)^2]^{1/2}$), which indicated the purity or intensity of the hue (McGuire, 1992).

2.3. Extraction and measurement of total anthocyanin

The total anthocyanin concentration was measured using a pH differential method and was presented as mg cyanidin-3-galactoside per 100 g fresh tissue (Dussi et al., 1995). One gram of fruit peel was mixed with methanol containing 0.01% HCl followed by centrifugation at 4 °C and 12,000 rpm for 20 min. The absorbance of each 100 μl extracted was assessed using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, U.S.) at 510 nm and 700 nm in buffers of pH 1.0 and 4.5. Anthocyanin content was calculated using $A = [(A_{510} - A_{700})\,pH_{1.0} - (A_{510} - A_{700})\,pH_{4.5}]$ with a molar extinction coefficient of cyanidin-3-galactoside of 3.02×10^4.

2.4. Assay of PAL and DFR activities

The method of PAL activity assay was described by Huang et al. (2009). PAL was extracted from 1 g of fruit peel by using 7.5 mL cold sodium borate buffer (200 mM, pH 8.8). The assay mixture contained 1 mL 50 mM L-phenylalanine and 0.8 mL crude enzyme in sodium borate buffer (200 mM, pH 8.8). After incubation at 37 °C for 90 min, 0.2 mL 6 M HCl was added to stop the reaction. The activity was assayed by measuring the absorbance of trans-cinnamic acid at 290 nm.

The method of Murray and Hackett (1991) was used for DFR extraction. 1 g of fruit tissue was ground briefly, washed with 2× 4 mL −20 °C acetone, and then extracted by the buffer containing 0.1 M borate buffer, pH 8.8, and 5 mM Ascorbic acid. The procedure of Stafford and Lester (1982) was used to assay DFR activity.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted using a modified CTAB method (Zhang X.D et al., 2011). The concentration of total RNA was measured after genomic DNA had been digested by DNase I. First-strand cDNA was synthesized from 4 μg of DNA-free RNA using a Revert Aid™ First-Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, U.S.). The